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**Abstract:** Liver function tests are of critical importance for the management of patients with severe or terminal liver disease. They are also used as prognostic tools for planning liver resections. In recent years many transport systems have been identified that also transport substances employed in liver function tests. Such substances include endogenous bilirubin or exogenously administered indocyanine green, agents for magnetic resonance imaging, agents for single photon emission computed tomography or agents for breath tests. The increasing functional and molecular information on the respective transport systems should improve the management and as a result the outcome of patients scheduled for liver surgery or transplantation. To achieve the latter goal, clinical studies that assess individual patients' liver function over the course of their disease with liver function tests are needed to firmly establish and validate recently introduced and novel liver function markers.

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# **The emerging role of transport systems in liver function tests**

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## **Abstract**

Liver function tests are of critical importance for the management of patients with severe or terminal liver disease. They are also used as prognostic tools for planning liver resections. In recent years many transport systems have been identified, that also transport substances employed in liver function tests. Such substances include endogenous bilirubin or exogenously administered indocyanine green, agents for magnetic resonance imaging, agents for single photon emission computed tomography or agents for breath tests. The increasing functional and molecular information on the respective transport systems should improve the management and as a result the outcome of patients scheduled for liver surgery or transplantation. To achieve the latter goal, clinical studies that assess individual patients liver function over the course of their disease with liver function testes are needed to firmly establish and validate recently introduced and novel liver function markers.

## **1. Introduction**

Prognostic tools are of critical importance for the management of patients with severe chronic liver disease or fulminant liver failure. They are also needed for planning liver resections in patients with hepatic malignancies. For the first situation, liver function tests and scoring systems have been established. In the latter case, functional liver volumetry is currently an important part of the assessment of patients prior to surgery (Vauthey et al., 2010). The classical scoring system used in patients with severe liver disease is the Child-Turcotte-Pugh score, which takes into account encephalopathy, ascites, bilirubin, albumin, and prothrombin time (Durand and Valla, 2008). This score is increasingly being replaced by the MELD score, which is calculated from plasma bilirubin, the international normalized ratio (INR), and plasma creatinine (Cholongitas et al., 2006). In both scores, plasma bilirubin is a parameter that monitors, among others, transport capacity of hepatocytes and biotransformation, i.e., the detoxifying capacity of the liver. Hepatocyte-mediated detoxification involves four phases: phase 0 represents the uptake of substances into hepatocytes, phase I metabolism of substances, phase II, conjugation and phase III export of substances and/or metabolites from hepatocytes (Petzinger and Geyer, 2006; Vavricka et al., 2002). Hence, transport proteins are key molecular components of the hepatocellular detoxification system (phase 0 and phase III) and consequently of liver function tests. Liver function tests utilizing exogenous indicators such as indocyanine green (ICG), bromosulphophthalein (BSP), fluorescent bile salt derivatives, agents for magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), or breath tests have been developed to complement the use of endogenous indicators (de Graaf et al., 2010a; Milkiewicz et al., 2000; Millet et al., 2011; Sakka, 2007). Preoperative dynamic liver function tests are part of the routine assessment of patients scheduled for liver resection in many centers (Breitenstein et al., 2009). Therefore, elucidation of the molecular mechanisms and especially the transport systems involved in dynamic liver function tests is highly warranted.

## **2. Transport systems in liver function tests**

### **2.1. Bilirubin**

Rat Oatp1a4 and human OATP1B1 and OATP1B3 (table 1) transport unconjugated or conjugated bilirubin (Briz et al., 2003; Cui et al., 2001b; Konig et al., 2000; Reichel et al., 1999). Corroboratively, recent pharmacogenetic studies have linked polymorphisms in the *SLCO1B1* and *SLCO1B3* genes with unconjugated and conjugated plasma bilirubin levels (Johnson et al., 2009; Sanna et al., 2009; Zhang et al., 2007). A role of Oatps in hepatocellular bilirubin uptake is further supported by experiments with mice with deleted *Slco1a* and *Slco1b* genes. These animals display a greater than 40-fold increase in total plasma bilirubin, the major part of which is conjugated bilirubin, which is not detectable in the parent strain (van de Steeg et al., 2010). Unconjugated bilirubin is increased approximately 2.5-fold. While the cause of the conjugated bilirubin elevation has not been determined, the increase in unconjugated bilirubin supports a role of Oatps in hepatocellular uptake of bilirubin. Mild elevation of mainly conjugated bilirubin is also observed in mice with inactivated *Slco1b2* (Zaher et al., 2008), which was recently confirmed (Csanaky et al., 2011). Taken together, these human and animal data strongly support a physiological involvement of hepatocellular OATPs in the hepatic uptake of unconjugated and conjugated bilirubin.

### **2.2. Bromosulphophthalein**

The first Oatp was isolated by expression cloning with BSP as substrate (Jacquemin et al., 1994). In the human liver, the three OATPs (table 1) are excellent BSP transporters (Hagenbuch and Meier, 2004; Kullak-Ublick et al., 2001). After uptake into hepatocytes, BSP is conjugated to glutathione and excreted into bile (Combes and Stakelum, 1960) by Mrp2 in rats (Takikawa et al., 1991). Unconjugated BSP is also a substrate for MRP2 (Cui et al.,

2001a) (table 1). Hence, BSP clearance monitors both, hepatocellular transport and metabolism. Today, BSP clearance is rarely measured in patients, as it may be associated with severe systemic reactions (Sakka, 2007).

### ***2.3. Indocyanine green***

Oatp1a1 as well as OATP1A2, the first cloned human OATP, are strongly inhibited by ICG (Bossuyt et al., 1996; Kullak-Ublick et al., 1995) (table 1). Recently, OATP1B3 and the human sodium-taurocholate cotransporting polypeptide (NTCP, SLC10A1) have been demonstrated to transport ICG (table 1), while OATP1B1 ( $IC_{50}= 3.2 \mu M$ ) and OATP2B1 ( $IC_{50}= 0.08 \mu M$ ) are strongly inhibited by ICG but do not transport ICG (de Graaf et al., 2011). ICG is not subject to hepatic metabolism (Wheeler et al., 1958). Eisai rats lacking functional Mrp2 reveal that biliary ICG excretion is mediated by Mrp2 (Sathirakul et al., 1993) (table 1). Delayed residual ICG excretion in these rats indicates an additional transcellular pathway for ICG. This view is supported by the observations that the microtubule disrupting agent colchicine partially inhibits biliary ICG excretion (Mori et al., 1987).

### ***2.4. Fluorescently labeled bile salts***

Reduced clearance of cholyl-glycine is a known phenomenon in patients with liver disease (LaRusso et al., 1975) and the clearances of cholyl-aurine and ICG correlate well (Paumgartner et al., 1979). Fluorescently labeled bile salts have been suggested as liver function indicators on the basis of a pilot study that demonstrated reduced clearance of cholyl-lysyl-fluorescein (CLF) in patients with liver cirrhosis (Milkiewicz et al., 2000). CLF is not a substrate of the major bile salt transporters NTCP and bile salt export pump (BSEP), but of OATP1B3, weakly of OATP1B1, and of the efflux transporters MRP2 and MRP3 (de Waart

et al., 2010) (table 1). Fluorescent chenodeoxycholic acid 7-nitrobenz-2-oxa-1,3-diazole is transported by OATP1B1 and OATP1B3 (Yamaguchi et al., 2006).

## ***2.5. Erythromycin***

The erythromycin breath test assesses the biotransformation function of the liver. Erythromycin inhibits OATP1B1 and OATP1B3 (Seithel et al., 2007), is transported by OATP1B1 (Franke et al., 2008) and is a substrate for MRP2 (Franke et al., 2011) (table 1). MRP2-mediated transport affects the metabolism of erythromycin in humans (Franke et al., 2011). Consequently, the interplay between transport and metabolism is of relevance for determining liver function by erythromycin.

## ***2.6. MRI and scintigraphic agents***

The MRI agent gadoxetate (gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid, Gd-EOB-DTPA) or gadobenate (gadolinium benzyloxypropionictetraacetate, Gd-BOPTA) are used to evaluate focal liver lesions by T1-weighted MRI imaging and more recently to evaluate liver function. Gadoxetate is excreted unaltered into rat bile (Weinmann et al., 1991) and its secretion into bile is almost completely blocked, when the animals are pretreated with BSP (de Haen et al., 1995). Rat Oat1a1, but not Oat1a4 or OATP1A2 (table 1), were found to transport gadoxetate (van Montfoort et al., 1999). Biliary gadoxetate excretion is massively reduced in TR<sup>-</sup> rats with no functional Mrp2 (de Haen et al., 1996). The structurally closely related Gd-BOPTA is taken up into rat hepatocytes by Oatp1a1, Oatp1a4, and Oatp1b2 and excreted into bile by Mrp2 (Planchamp et al., 2007). Recently, the scintigraphic agent <sup>99m</sup>Tc-mebrofenin was introduced for assessing liver function noninvasively (de Graaf et al., 2010a) and for predicting the function of the future remnant liver after liver resection (de Graaf et al., 2010b). Subsequent studies identified OATP1B1 and OATP1B3 as <sup>99m</sup>Tc-mebrofenin uptake transporters (de Graaf et al., 2011; Ghibellini et al., 2008). Biliary excretion of <sup>99m</sup>Tc-

mebrofenin is mediated by MRP2 and basolateral efflux by MRP3 (Ghibellini et al., 2008) (table 1).

### **3. Impact of transport processes on liver function tests**

#### ***3.1. Effect of dose of the indicator***

As transport systems exhibit saturation, the disappearance of the indicator from the blood may - depending on its dose - follow simple or complex kinetics. Thus, the plasma concentration of the indicator following administration may affect its kinetics. This phenomenon has been worked out in detail for ICG. This prototypic indicator is almost exclusively eliminated by the liver and can be used for monitoring hepatocellular transport function as it is not metabolized. At low doses, ICG behaves as a high extraction drug. Consequently its plasma clearance is mainly determined by liver blood flow and it is not a sensitive parameter of the liver excretory functional capacity. At high doses, ICG displays saturation of the clearance and mainly reflects the transport capacity of the liver (Paumgartner, 1975).

#### ***3.2. Importance of transport mechanism***

The type of transport mechanism will affect the clearance of the liver function indicators. For example, NTCP is a secondary active, electrogenic transporter that mediates substrate uptake by utilizing the electrochemical sodium gradient across the basolateral plasma membrane of hepatocytes (Stieger, 2011). This driving force is maintained by the (Na+K)-ATPase, which requires a continuous supply of ATP.

The transport mechanism(s) of OATPs is not fully understood (Hagenbuch and Gui, 2008). They are believed to act as organic anion exchangers with bicarbonate (Satlin et al., 1997) and/or reduced or oxidized glutathione (Briz et al., 2006; Li et al., 1998; Li et al., 2000) acting



as counterions. Recently, evidence was presented that the activity of practically all OATPs is stimulated by a low extracellular pH and that many OATPs act as anion-exchangers with bicarbonate as counter ion (Leuthold et al., 2009). Hence, while anion exchange as basic transport mechanism of OATPs seems well established, clearly more work is needed to obtain detailed insights.

The transport mechanism will influence the capacity of OATPs to take up liver function indicators into hepatocytes. If OATPs can work against a concentration gradient, the consequence will be an intracellular concentration exceeding the plasma concentration. ICG has been found at approximately 10-fold higher concentration in rat liver than in plasma (Horak et al., 1973). Similarly, glibenclamide is 50 times higher concentrated in rat livers than in plasma (Kellner et al., 1969). Glibenclamide inhibits OATP1B1 and OATP1B3, making it a likely substrate for OATPs (Bednarczyk, 2010). Hepatocellular concentrations of liver function indicators are not only determined by uptake into hepatocytes but also by the relative rate of export, as recently demonstrated for Gd-BOPTA (Millet et al., 2011). Perfusing wild type and TR<sup>-</sup> rat livers with increasing concentrations of Gd-BOPTA showed unaltered  $K_m$  of hepatic uptake and only a 25 % reduction in  $V_{max}$  in TR<sup>-</sup> compared to wild type rats despite a lack of biliary secretion in the mutant rats. Hence, the interplay of uptake and export is intricate. Complex interplay between uptake and efflux was also demonstrated for fexofenadine and napsagatran (Poirier et al., 2009).

### ***3.3. Interindividual variability of transporter expression***

Transporter expression displays a considerable interindividual variability (Ohtsuki et al., 2011). In healthy liver tissue from 110 individuals, protein expression of the four canalicular transporters BSEP, MDR3, MRP2 and MDR1 displayed a considerable, up to 33-fold,

interindividual variability (Meier et al., 2006). Such interindividual differences in transporter expression levels are expected to affect the clearance of liver function indicators.

### ***3.4. Transporter expression in liver disease***

The expression of hepatocellular transporters may be altered divergently in liver disease. For instance, progressive familial intrahepatic cholestasis leads to downregulation of NTCP, OATP1B1, OATP1B3, and MRP2, while MRP4 is upregulated (Keitel et al., 2005). Patients with inflammatory cholestasis, primary biliary cirrhosis or chronic hepatitis C show a reduction of protein expression for NTCP and OATP1B1 (Zollner et al., 2003; Zollner et al., 2001), while expression of MRP3, MRP2, MDR1, and MDR3 is upregulated and BSEP is not affected (Zollner et al., 2003). Hence, clearance prediction of liver function indicators is difficult in disease situations. The alteration of transporter expression is not always evenly distributed throughout the liver. For instance, some hepatocellular carcinomas display poor MRI enhancement with gadoxetic acid. The enhancement correlates positively with OATP1B1 and OATP1B3 and negatively with MRP2 expression (Tsuboyama et al., 2010).

### ***3.5. Interaction of drugs with transport systems***

Drugs that inhibit transporters of liver function indicators may influence liver function tests. For example, uptake of ICG is inhibited by rifamycin (Paumgartner, 1975), which is a potent liver OATP inhibitor (Fattinger et al., 2000; Vavricka et al., 2002). In contrast, co-administration of erythromycin and gadoxetic acid was recently found to have no effect on liver imaging (Huppertz et al., 2011).

## **4. Perspective**

Taken altogether, it is now well established that transport systems are key elements in most, if not all currently used liver function tests. Consequently, read outs from liver function tests

represent a complex interplay between uptake of the indicator, its potential metabolism, and its bidirectional export from hepatocytes. This complexity of the monitored physiological and/or pathophysiological processes makes it highly likely that one single test of liver function is of limited predictive value. The challenge will now be to delineate in prospective studies a set of liver function tests to be applied in clinics for predictive purposes.

One potential patient collective to be addressed is for example patients on the waiting list for organ transplantation, who are currently prioritized by means of the MELD score and by changes in the MELD score. However, certain diseases underlying liver failure necessitate exceptions from the MELD score (Massie et al., 2011). In such cases, novel markers of global liver function are needed and should be compared with the MELD score in individual patients. An important criterion will be that such a novel marker performs better than the ICG clearance test, which is known to suffer from inaccuracy and limited reproducibility (Schneider, 2004). In regard to the MELD score, it should also be borne in mind that patients with cirrhosis potentially present with factors that lead to falsely low plasma creatinine values, which will overestimate the true glomerular filtration rate (Sherman et al., 2003). This will lower their MELD score and hence potentially lead to a too low priority rating on waiting lists. Again, novel global liver function tests may be a solution here.

In the case of planned liver resections, information on the liver function of individual segments is needed to successfully improve the outcome of this procedure (de Graaf et al., 2010a; Hoekstra et al.). Noninvasive methods such as scintigraphy or MRI are already under clinical investigation and have demonstrated to predict the remaining functional liver mass in patients scheduled for resection (de Graaf et al., 2010c). Future studies should be designed such that they yield a clear definition and grading of posthepatectomy liver failure, which is currently not classified by a generally accepted scoring system (Rahbari et al., 2011). In all

instances, future clinical studies should certainly involve longitudinal studies in individual patients, e.g., those listed for transplantation to overcome inherent limitations such as interindividual differences in transporter expression (Meier et al., 2006; Ohtsuki et al., 2011). Longitudinal studies will be particularly attractive with tests that can be monitored transcutaneously in a given patient, e.g., ICG clearance (Sakka, 2007) or  $^{99m}\text{Tc}$ -mebrofenin (de Graaf et al., 2010b).

Given the current knowledge on the role of transport proteins in the molecular mechanisms underlying liver function tests, more information on the function and expression of these transporters is needed. In this respect, quantitative proteomics has become a highly attractive tool for the determination of transporter expression levels in individual patients (Ohtsuki et al., 2011). A possible scenario could involve the intraoperative determination of the clearance of a liver function marker with simultaneous proteomic determination of transporters in the resected liver tissue. A proof-of-principle for such an approach has now been established for transporter expression in the blood-brain barrier in an animal model (Ito et al., 2011). Finally, the successful establishment of tests to reliably predict the functional capacity of the future remnant liver will hopefully improve the management and, as a result, the outcome of patients undergoing liver resection.

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Table 1: Human transporters involved in the transport of liver function indicators

Transporter	Protein Name	Synonym	Gene Symbol	expressed in hepatocytes	liver function indicator	functional variants known
OATP1A2	organic anion transporting polypeptide	OATP, OATP-A	SLCO1A2	?	BSP, gadobenate	yes <sup>1</sup>
OATP1B1	organic anion transporting polypeptide	OATP2, OATP-C, LST-1	SLCO1B1	yes	bilirubin, BSP, CLF, erythromycin, mebrofenin	yes
OATP1B3	organic anion transporting polypeptide	OATP8	SLCO1B3	yes	bilirubin, BSP, ICG, CLF, mebrofenin	yes
NTCP	sodium taurocholate cotransporting polypeptide		SLC10A1	yes	ICG	yes <sup>2</sup>
MRP2	multidrug resistance-		ABCC2	yes	BSP, ICG (rat Mrp2), CLF,	yes

	associated protein				erythromycin, mebrofenin	
MRP3	multidrug resistance- associated protein	cMOAT	ABCC3	yes	CLF, mebrofenin	yes

Extensive information on the known functional variants of the transporters can be found in (Stieger and Meier, 2011) except for <sup>1</sup>(Kalliokoski and Niemi, 2009) and <sup>2</sup>(Stieger, 2011).

BSP: bromosulphophthalein, CLF: cholyl-lysyl-fluorescein, ICG: indocyanine green